

Biomarkers of immunosuppressive drug toxicity

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Purpose of review

Chronic immunosuppressant toxicity plays a key role in chronic nephropathy after transplantation. Whereas acute toxicity is usually associated with increased drug exposure and can reliably be detected using pharmacokinetic drug monitoring, detecting chronic toxicity is more challenging. Pharmacokinetic therapeutic drug monitoring alone does not appear to be a viable strategy to avoid chronic immunosuppressant toxicity and the development of new techniques is warranted. Modern screening technologies in the fields of genetics, genomics, protein profiling (proteomics), and biochemical profiling (metabonomics) have opened new opportunities for the development of sensitive and specific diagnostic tools. In this review, we summarize the status of biomarker development in transplantation with an emphasis on toxicodynamic monitoring.

Recent findings

Most clinical studies relevant for immunosuppressant toxicity have been carried out in the field of pharmacogenetics and toxicogenetics. The goal has been the prediction of an appropriate initial dose and thus avoidance of early under-immunosuppression and acute toxicity. The importance of utilizing pharmacogenetics, toxicogenetics, proteomics, and metabonomics for establishing assays to monitor and distinguish chronic rejection and chronic immunosuppressant toxicity has not yet been fully explored.

Summary

Toxicogenetics, toxicogenomics, proteomics, metabonomics, and integrated systems approaches are promising strategies for the development of biomarker-based toxicodynamic monitoring of transplant patients.

Keywords

biochemical profiling, calcineurin inhibitors, chronic allograft dysfunction, everolimus, immunosuppressant toxicity, metabolomics, metabonomics, mycophenolic acid, pharmacogenetics, proteomics, sirolimus, toxicogenomics

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Abbreviations

¹H-MRS	hydrogen-magnetic resonance spectroscopy
MRS	magnetic resonance spectroscopy
TMPT	thiopurine methyltransferase

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Introduction

A biomarker is defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological response to therapeutic intervention’ [1]. Biomarkers are used to study, monitor, and diagnose disease processes. Indicative of the broadness of the definition, the measurement of biomarkers encompasses a wide variety of methodologies ranging from imaging technologies to gene arrays. In fact, clinical diagnostics must be considered as nothing less than the use of biomarkers [2*]. Also, the term biomarker has the unfortunate implication that the goal is to identify one specific parameter that is closely correlated with a specific disease process. This is true for only a few biomarker–disease combinations. In general, specific biomarker patterns will confer significantly more information than a single measurement and enable better specificity and sensitivity [2*,3*]. An ideal case scenario for diagnostic testing is the use of readily available body fluids such as plasma and urine for biomarker analysis. This is based on the concept that all cells directly or indirectly (via extracellular fluid) communicate with body fluids and that cell metabolites, peptides and proteins will be released by the cells via normal excretion, transmembrane diffusion and transport as well as after cell death [4*]. Changes in protein, peptide, and metabolite patterns in body fluids to a certain extent will be reflective of intracellular changes.

Immunosuppressant toxicity and long-term outcome after transplantation

Recent epidemiological studies have shown that, although current immunosuppressive protocols have dramatically decreased and almost eradicated acute rejection episodes, there has been very little progress in long-term graft survival after kidney transplantation over the last two decades [5,6**,7*]. While cardiovascular complications are the major cause of death in kidney transplant patients [8], chronic renal allograft dysfunction is the principal cause of late renal allograft loss after the first year [8]. Advanced donor age, cold ischemia time, acute rejection, and exposure to immunosuppressants have been

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identified as independent major risk factors [7[•],9,10[•],11]. Progressive, chronic allograft dysfunction is also a problem after other types of solid organ transplantation [12,13], as well as chronic kidney dysfunction in nontransplant patients that are treated with immunosuppressants such as calcineurin inhibitors.

The important contribution of calcineurin inhibitor toxicity to chronic renal allograft dysfunction was recently documented by Nankivell *et al.* who observed that histologic changes indicating cyclosporine toxicity such as hyalinosis were universally found in cyclosporine-treated recipients [14^{••}]. Another problem is the high incidence of immunosuppressant-induced cardiovascular deaths observed in patients with a functioning transplant [6[•],8]. Immunosuppressants have a significant effect on endothelial function including oxidative stress, apoptosis, metabolic changes, and changes of released vasoactive factors [15[•]].

The key to reducing or avoiding the negative effects of chronic rejection and immunosuppressant toxicity is early detection. Once detected, damage by chronic rejection can be reduced by immunologic intervention [16[•]] and in the case of immunosuppressant toxicity, modification of the immunosuppressive drug regimen [7[•]]. Sensitive and specific diagnostic tools that can distinguish between chronic rejection and chronic immunosuppressant toxicity and that assist in the management of transplant patients are clearly lacking. An attractive new strategy is the identification of molecular 'signatures' in the transplant organ, blood, or plasma [6[•],17[•]], which will allow for monitoring immunologic organ damage and immunosuppressant toxicity. Indeed, modern screening technologies in the fields of genetics, genomics, protein profiling (proteomics), and biochemical profiling (metabonomics) allow for an unbiased, holistic or 'systems biology' approach to the effects of disease, drugs, and environment on the human body. In this review, we will summarize the status of using biomarker development based on proteomics, metabolomics, genomics, and genetics in transplantation (Table 1) with emphasis on the monitoring of immunosuppressant toxicity.

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Pharmacogenetics and pharmacogenomics

Within the field of pharmacogenetics, several areas highlight the contributions of this emerging discipline to the determination of immunosuppressant toxicity: genetic disposition, regulation of biomolecules, gene arrays as a means of identifying biomarkers, and the toxicogenetics and toxicogenomics of specific immunosuppressants. For example, the genetic regulation of specific classes of molecules such as cytokines [18[•],19,20[•]], cytokine receptors, co-stimulatory molecules, adhesion molecules, and chemokine gene expression are increasingly recognized as potential prognostic marker for long-term outcome after

transplantation. This indicates that immunogenetic variability influences allograft survival and outcome beyond affecting MHC molecules [21,22,23[•],24]. Detailed lists of gene variants of relevance are provided in the references [22,23[•],24]. In transplantation, the genomics of both donor and recipient are of importance [25,26[•]]. Also, the distribution of polymorphisms differs between ethnic groups [27]. In terms of chronic renal allograft dysfunction, analysis of cortical mRNA has successfully been used to distinguish between chronic rejection and chronic cyclosporine toxicity in renal allograft patients [28[•]]. mRNA levels of laminin β_2 and transforming growth factor- β (TGF- β) were significantly higher in patients with chronic cyclosporine toxicity. Using a 15.6-fold increase cut-off, laminin β_2 mRNA expression predicted cyclosporine toxicity with 87% sensitivity and 88% specificity [28[•]].

It has become apparent that a link exists between polymorphisms and toxicity and interest has now focused on the pharmacokinetics and toxicogenetics of immunosuppressants [29[•]]. Drug metabolizing enzymes and drug transporters are involved in the pharmacokinetics of most immunosuppressants. The pregnane X receptor and the constitutive androstane receptor regulate expression of several drug metabolizing enzymes and drug transporters that are of relevance for immunosuppressant pharmacokinetics [30[•]]. For immunosuppressants, the clinically most relevant drug metabolizing enzymes are cytochrome P450 3A4 (CYP3A4) and 3A5 (CYP3A5) as well as the ATP-binding cassette transporter p-glycoprotein [30]. Genetic polymorphisms are established for most of the drug metabolizing enzymes (<http://www.imm.ki.se/CYPalleles>), drug transporters, and nuclear receptors [30[•]]. It is reasonable to expect that these polymorphisms contribute to the pharmacokinetic and pharmacodynamic variability of immunosuppressants, and thus to immunologic risks and their toxicity [29[•],31[•],32]. The promising role of pharmacogenetics and toxicogenetics as well as pharmacogenomics and toxicogenomics lies in the evaluation and prediction of the most efficient and tolerable immunosuppressive drug regimen [29[•],31[•],32]. Azathioprine was the first immunosuppressant for which the correlation between gene polymorphisms and toxicity was recognized. Thiopurine methyltransferase (TMPT) is a key enzyme in azathioprine metabolism. When patients with low activity receive normal doses, they develop drug-induced myelosuppression. The gene variants *TMPT**2-13 are associated with low TMPT activity [33–35]. Phenotypic testing of red blood cells and DNA-based tests for *TMPT* were among the first pharmacogenetic tests used in clinical practice [29[•]]. In liver transplant patients, *TMPT* gene variants assessed in the recipients' blood were not correlated with myelo-suppression [36[•]]. Azathioprine is mostly metabolized in the liver and thus donor genetics seems to be more relevant for azathioprine pharmacokinetics after liver transplantation.

Table 1. Modern screening technologies used to identify and develop biomarkers

Technology	Principle	Assays	Status	Limitations as diagnostic tools
Pharmacogenetics, toxicogenetics	Analysis of single nucleotide polymorphisms (SNPs)	PCR, chips/arrays, MALDI/TOF	Well-established, high-throughput technologies	<ul style="list-style-type: none"> - Genetics is static; effects by disease, drug and/or environment are not reflected, - When interactions between drug-metabolizing enzymes, drug transporters and drug targets are complex and/or not completely understood, the predictive value may be limited. - mRNA concentrations may not correlate with the concentration of active protein - will require biopsies.
Genomics, toxicogenomics	mRNA expression analysis	Chips/arrays, PCR	Well-established, high-throughput technologies	<ul style="list-style-type: none"> - The proteome is extremely complex and constantly in flux, - The proteome is only partially identified and not all potentially relevant proteins may be detected, - The range of proteins detected depends on the methodology used, - Sometimes poor reproducibility and database searches result in multiple hits, validation of search results may be required.
Proteomics	Analysis of protein expression, protein identification	2D gel electrophoresis, multidimensional LC/MS, MALDI-TOF, SELDI-TOF, CE-MS, protein chips, LC-MS in combination with database searches	<ul style="list-style-type: none"> - Many different technologies, some of which are still under development, - Quantification and validation is still a challenge 	<ul style="list-style-type: none"> - Only about 25% of the metabolites found in plasma are structurally identified, - Specificity may be lower than proteomics/genomics since many diseases result in similar metabolic changes, e.g. diseases or drug toxicities that cause oxidative stress, - Specific information may be encoded in pattern changes of low-concentration metabolites that are below the lower limit of detection.
Metabonomics	Measurement/quantification of cell metabolites in tissues and body fluids	LC-MS, CE-MS, GC-MS, MRS	<ul style="list-style-type: none"> - For LC-MS using electrospray interfaces ion-suppression is a still underestimated and largely unsolved problem - Databases for metabolite identification for LC-MS and CE-MS are just emerging - No established assay validation procedures. 	<ul style="list-style-type: none"> - Instrumental and bioinformatics analyses as well as validation are of high complexity, - Will require a complex infrastructure and data management system, - Semantic or nomenclature standards not yet available that will allow for combining results and data from different sources.
Systems biology, systems toxicology	All of the above	All of the above	<ul style="list-style-type: none"> - Requires simultaneous investigation of all interacting components. This is a hypothetical advantage over more limited approaches, but not all the necessary tools are in place yet: - Integrative bioinformatics approaches for systems biology analyses are just developing, - No established assay validation procedures. 	

Abbreviations: CE, capillary electrophoresis; GC, gas chromatography; LC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; SELDI, surface-enhanced laser desorption ionization; TOF, time-of-flight mass spectrometry.

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Table 2. Prediction of immunosuppressant pharmacokinetics and dosing by CYP3A4, CYP3A5 and ABCB1 (P-glycoprotein) gene polymorphisms

Patients	Drug (PK parameters)	Polymorphisms		Comments	Ref.
		Relevant for prediction	Irrelevant for prediction		
Renal transplant patients	Cyclosporine (dose adjusted through blood concentration)	CYP3A4, CYP3A5	CYP3A4, CYP3A5, ABCB1 genotypes		[37]
Renal and heart transplant patients	Cyclosporine, (apparent oral clearance, C _l /F)		CYP3A4, CYP3A5, ABCB1 genotypes	A population pharmacokinetic approach was used, CYP3A4*1B variant allele carriers had a higher oral cyclosporine clearance than CYP3A4*1. However, this difference was not considered clinically relevant.	[38]
Renal transplant recipients	Cyclosporine (dose adjusted through blood concentration)	CYP3A5	ABCB1 genotypes	CYP3A5*1/*3 carriers required 1.6-fold higher doses to achieve the same blood concentrations as CYP3A5*3/*3 carriers.	[39]
Renal transplant patients	Cyclosporine, (dose adjusted AUC _{0-4h} , AUC _{0-12h} , and C _{max})		CYP3A5, ABCB1 genotypes	ABCB1 haplotype analysis showed a trend towards higher AUC _{0-4h} for the carriers of the T-T haplotype (exons 12, 21, 26), however, this was not considered clinically relevant.	[40]
Liver transplant patients	Cyclosporine (dose adjusted concentration at 2 hours C2)	ABCB1 3435		ABCB1 exon 26 C3435T in the liver recipient is a major determinant for cyclosporine concentrations (C2)/dose ratios	[41]
Heart transplant patients	Cyclosporine, (AUC _{0-4h} , AUC _{0-12h} , C _{max} , through blood concentration)	ABCB1 haplotypes	CYP3A4	The results suggest that ABCB1 haplotypes rather than genotypes influence cyclosporine pharmacokinetics. T-T haplotype (exons 12, 21, 26) carriers had higher cyclosporine exposure than C-G-C haplotypes.	[42]
Renal transplant patients	Sirolimus, (dose adjusted through blood concentration)	CYP3A4, CYP3A5	ABCB1 genotypes	Patients with CYP3A4*1B or CYP3A5*1 haplotypes required significantly higher doses to achieve adequate blood through concentrations. The study confirmed the hypothesized linkage between CYP3A5*1 and CYP3A4*1B allele polymorphism.	[43]
Renal transplant patients	Tacrolimus, (dose adjusted through blood concentration)	CYP3A5		The tacrolimus doses required in CYP3A5*1/*1 carriers to achieve tacrolimus blood through concentrations in the target range was significantly higher than in CYP3A*3/*3 carriers. However, no significant difference was found when compared to CYP3A5*1/*3 carriers.	[44]
Renal transplant patients	Tacrolimus, (dose adjusted through blood concentration)	ABCB1 genotypes and haplotypes		The data suggested that exon 26 and 21 SNPs allow for predicting tacrolimus dose requirements	[45]
Renal transplant patients	Tacrolimus, (dose adjusted through blood concentration)	CYP3A4, CYP3A5		Patients with at least one CYP3A5*1 allele required twice the tacrolimus dose to achieve target blood concentrations and showed a significant delay in achieving those target concentrations.	[46]
Renal transplant patients	Tacrolimus, (dose adjusted through blood concentration)		ABCB1 genotypes	CYP3A5*3/*3 carriers require less tacrolimus than carriers of other CYP3A5 genotypes, CYP3A4*1 homozygotes require less tacrolimus than CYP3A4*1B carriers.	[37]
Renal transplant patients	Tacrolimus (dose adjusted through blood concentration)	CYP3A5	ABCB1 genotypes	CYP3A5*1/*3 carriers required threefold higher doses to achieve the same blood concentrations as CYP3A5*3/*3 carriers.	[39]
Renal transplant patients	Tacrolimus, (dose adjusted concentration-time curve, AUC _{0-12h})	CYP3A5	ABCB1 3435	CYP3A5*1 carriers require a higher dose than CYP3A5*3/*3 carriers	[47]
Living donor related liver transplant recipients and donor livers	Tacrolimus, (dose adjusted through blood concentration)	CYP3A5 in the donor liver	ABCB1 genotypes	ABCB1 in the recipient may play a role during the first week, hereafter, liver metabolism and thus the donor genotype plays the key role for tacrolimus elimination	[48*]
Lung transplant patients	Tacrolimus, (dose adjusted through blood concentration)	ABCB1 2677 haplotypes	ABCB1 3435 haplotypes	The authors conclude that ABCB1 haplotype analysis may be of clinical relevance	[49*]

AUC, area-under-the-time-concentration curve; C₂, cyclosporine concentration 2 hours after administration; C_l, clearance; C_{max}, maximum blood concentration; F, oral bioavailability; PK, pharmacokinetics; ref, reference; SNP, single nucleotide polymorphism.

Table 3. Identification of proteomics-based biomarker for diagnosis or monitoring after transplantation

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Ref.
Acute renal allograft rejection	17 patients with and 15 patients without rejection	Urine	SELDI-TOF	<ul style="list-style-type: none"> - 5 Polypeptides with 6.5, 6.6, 6.7, 7.1 and 13.4 kDa were identified that allowed for classification, - Those peptides/proteins were not further identified, - Sensitivity 83%, specificity 100% 	[64]
Acute renal allograft rejection	19 patients with different grades of rejection (Baniff 1997 Ia to IIb), 10 patients with urinary tract infection, 29 patients without rejection, 66 nontransplant subjects	Urine	CE-TOF	<ul style="list-style-type: none"> - 17 Urinary polypeptides discriminated between renal transplant patients and nontransplant patients, - 10 between urinary tract infection and samples without infection or rejection (control), - 16 between renal allograft patients with and without rejection, - 10 between acute allograft rejection and urinary tract infection, - One protein differentiating between healthy subjects and renal allograft patients, a fragment of collagen alpha 5(IV) protein, was identified. 	[65*]
Acute renal allograft rejection	23 patients with and 22 patients without rejection, 20 healthy subjects	Urine	SELDI-TOF	<ul style="list-style-type: none"> - 7 Polypeptides with 2.0, 2.8, 4.8, 5.9, 7.0, 19.0 and 25.7 kDa were identified that allowed for classification, - Acute rejection could be distinguished from stable renal allograft patients with sensitivity of 90.5–91.3% and a specificity of 77.2–83.3%, - A protein with the 78.5 kDa was found that distinguished between renal allograft patients and healthy subjects. Sensitivity and specificity were 100% 	[66*]
Acute renal allograft rejection	18 patients with and 22 patients without rejection, 5 patients with tubular necrosis, 5 patients with glomerulo-pathy, 5 nontransplant patients with urinary tract infections, 28 healthy subjects	Urine	SELDI-TOF	<ul style="list-style-type: none"> - Patients with rejection showed prominent peak clusters in regions of m/z = 5270–5550, 7050–7360, and 10530–11100, - In urine from normal subjects, those clusters were missing, - 82% from the stable transplant group and 6% from the acute rejection group did not show those clusters, - The peptides/proteins in the clusters were further structurally identified in reference [68*] and were found to be mostly associated with β2-microglobulin. 	[67*]
GVHD after hematopoietic stem-cell transplantation	40 transplant patients, 5 patients with sepsis	Urine	CE-TOF	<ul style="list-style-type: none"> - 21 Polypeptides were identified that allowed for classification between transplant patients and healthy subjects (95% sensitivity, 100% specificity) - There was no difference caused by the conditioning regimens, - 16 polypeptides distinguished patients with from those without GVHD and from those with sepsis (82% specificity/100% sensitivity), - Two of the polypeptides indicative of GVHD were sequenced and identified as a leukotriene A4 hydrolase fragment and a serum albumin fragment. 	[69*]

GVHD, graft-versus-host disease; CE, capillary electrophoresis; SELDI, surface-enhanced laser desorption ionization; TOF, time-of-flight mass spectrometry.

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T2 The most important pharmacogenetic clinical studies assessing the impact of polymorphisms of *CYP3A4*, *CYP3A5*, and P-glycoprotein (*ABCB1*) genes on the pharmacokinetics of immunosuppressants are summarized in Table 2 [37–47, 48•, 49•]. In summary, studies evaluating the effect of *CYP3A* and *ABCB1* polymorphisms on cyclosporine pharmacokinetics show that *ABCB1* haplotypes seem to be better predictors than *CYP3A* gene variants [29•,50]. This is not surprising because a clinical trial demonstrated that P-glycoprotein in the small intestine, but not *CYP3A*, significantly contributes to inter-patient variability in cyclosporine pharmacokinetics [51]. This can potentially be explained by the fact that orally administered cyclosporine undergoes significant intestinal metabolism and that P-glycoprotein-mediated efflux controls cyclosporine concentrations in the mucosa cells and thus access of cyclosporine to intestinal *CYP3A* [30•]. In contrast, *CYP3A5* polymorphism was a better predictor of tacrolimus pharmacokinetics than *ABCB1* [50]. Overall, predicting pharmacokinetics for immunosuppressants (Table 2) is complex and variants of other relevant genes such as additional transporters and nuclear receptors as well as the affinity of drugs to drug metabolizing enzymes and transporters must be taken into account. Also, transplant patients receive a multitude of drugs and drug–drug interactions may further complicate relevant predictions of clinical immunosuppressant pharmacokinetics and dosing [30].

The pharmacodynamic and toxicodynamic effect of a drug is determined by its concentration at the effector site, which is governed by pharmacokinetics, tissue distribution, and intracellular distribution. Active transporters may regulate the access of drugs to the target tissues as well as cellular compartments. There is a considerable extent of variation in the sensitivity of individual lymphocytes to each immunosuppressive drug alone or in combination, which pharmacokinetic monitoring cannot account for [52]. Lymphocytes also express ATP-binding cassette transporters such as P-glycoprotein that is encoded by the *ABCB1* gene. It has been hypothesized that overexpression of P-glycoprotein may be one of the reasons for acute and chronic rejection episodes despite adequate tacrolimus or cyclosporine blood concentrations [53]. The variability in the expression of P-glycoprotein in lymphocytes seems to be an important factor that reduces the correlation between immunosuppressant pharmacokinetics and its immunosuppressive activity. Interestingly, the *ABCB1* polymorphisms *G2677T* in exon 21 and *C3435T* in exon 26 do not seem to affect P-glycoprotein activity in CD56+ and CD4+ peripheral blood lymphocytes [54].

As mentioned above, active transport of immunosuppressants plays a critical role in tissue distribution of immunosuppressants and thus also in the toxicodynamics of immunosuppressants. In a study in kidney transplant

patients it was found that the *ABCB1 3435TT* genotype, which results in low expression of P-glycoprotein in the donor kidney was strongly associated with cyclosporine nephrotoxicity [55•]. Cyclosporine doses and exposure were not different from patients with other *ABCB1 3435* genotypes in the transplant kidney who did not show nephrotoxicity. Those results suggested that P-glycoprotein expression in the transplant kidney plays a significant role in cyclosporine nephrotoxicity. Also, the mutation in *ABCB1* at position 2677 in exon 21 in the recipient seems to increase the risk for tacrolimus-induced neurotoxicity [56]. P-glycoprotein is a key component of the blood–brain barrier that actively pumps drugs back into the blood [57]. Overall, those studies indicate that high expression levels of *ABCB1* in lymphocytes may reduce immunosuppressive efficacy, but on the other hand, high levels in the transplant kidney and blood–brain barrier may be protective against calcineurin inhibitor toxicity.

The most comprehensive genomic approach to profiling kidney transplant patients was reported by Flechner *et al.* [58••]. The investigators used commercial gene microarrays to determine gene expression profiles for kidney biopsies and peripheral blood lymphocytes. Unique gene expression signatures distinguishing acute rejection, acute graft dysfunction without rejection, and normal graft function could be identified [58••]. Strategies such as these may lead to integrative algorithms that take immunogenomics, pharmacogenomics, and toxicogenomics aspects and interactions into account.

Proteomics

Today, proteomics comprises the following four applications: protein mining, protein network mapping, mapping of protein modifications, and protein expression profiling [59•]. The latter is currently of most interest for the development of clinical diagnostic tools. The field of proteomics has mainly been driven by advances in mass spectrometry, databases, computing power, and informatics. Proteomic technologies allow for the simultaneous analysis of thousands of low-molecular-weight proteins, which may reveal patterns of disease, and are potentially useful for detection and assessment of prognosis [60•]. Proteomics has generated most interest in the field of cancer [60•]; however, several clinical studies have focused on the identification of protein patterns for the diagnosis of kidney diseases [61,62•,63•]. The most important studies assessing the proteome in transplant patients during rejection or graft-versus-host disease are summarized in Table 3 [64,65•,66•,67•,68•,69•]. In all studies listed, urine was the matrix of choice. A common problem with plasma and serum is that they contain high-abundance proteins such as albumin or immunoglobulins. These proteins can mask the most diagnostically valuable information which is conferred by mid- and low-abundance proteins [4•]. Although technology to remove high-abundance plasma

proteins exists, urine is attractive for the following reasons: Its collection is non-invasive and the kidney serves as a molecular filter and removes most of the high-abundance proteins making sample preparation less labor-intensive. Another aspect is that metabolites and proteins released into the blood will be diluted, while the metabolites in urine are concentrated by the kidney. This is especially relevant if the diagnostic target organ is the kidney itself. The most frequently used technologies for developing proteomics-based diagnostic tools in transplantation are surface-enhanced laser desorption ionization – time-of-flight mass spectrometry [70•] and capillary electrophoresis time-of-flight mass spectrometry [71]. Further developments in the area of protein microarrays and biochips may generate interesting high-throughput alternatives [72•]. As is apparent from Table 3, there seems to be little consistency regarding the peptides and proteins of diagnostic interest that were identified in those studies assessing the urine proteome of renal allograft patients with acute rejection. Potential reasons are that different technologies will preferentially detect different peptides and proteins and differences exist in sample preparation methods, such as the choice of chip surfaces for surface-enhanced laser desorption ionization – time-of-flight mass spectrometry analysis. Also, factors such as differences in the selection criteria for assigning transplant patients to groups with and without rejection as well as the relatively small numbers must be taken into account. A key in identifying valid parameters is the knowledge of the effect of gender, age, genetic and ethnic background, nutrition, and environment on the proteome. Although all studies in Table 3 included healthy subjects, the numbers (less than 100) were relatively small and may not be representative. Development of reference databases for normal subjects such as initiated by the Human Proteome Organization [73•] is critical. The only study using a proteomic approach to assess immunosuppressant nephrotoxicity was reported by Aicher *et al.* [74]. This study used two-dimensional gel electrophoresis analysis of kidney biopsies and demonstrated a marked decrease of calbindin-D 28kDa protein concentrations in patients with cyclosporine toxicity.

As of today, proteomic studies and diagnostic tool development in transplantation have clearly focused on acute rejection and those studies have shown great promise. Very little to no information is currently available regarding proteomic patterns that allow for monitoring chronic rejection, allograft dysfunction, and immunosuppressant toxicity.

Metabonomics

Metabonomics is defined as ‘a quantitative measurement of multi-parametric metabolic responses of multi-cellular systems to pathophysiological stimuli or genetic signaling’ [75]. The major technologies used are magnetic resonance spectroscopy (MRS) and mass spectrometry [76–78]. The

advantage of mass spectrometry is its superior sensitivity and the advantage of MRS is its noninvasiveness [76–79•]. Metabonomics has a long history in toxicology [80] and one of the foci has been nephrotoxicity [81]. Reference [81] includes a detailed list of metabolic markers associated with various toxins as determined via metabonomic analysis. Metabonomic profiling of the effects of immunosuppressants alone and in combination has mainly been based on ex-vivo and animal models. Those studies are summarized in references [79•,82•,83]. A study in rats treated with oral cyclosporine for 9 days clearly identified metabolic pattern changes in urine using hydrogen-magnetic resonance spectroscopy (¹H-MRS), high-performance liquid chromatography-mass spectrometry, and pattern recognition technologies [84•]. Changes of metabolic patterns in blood after treatment of nontransplant rats with cyclosporine, sirolimus, and/or everolimus for 6 days were detected using ¹H-MRS [79•]. After transplantation, ¹H-MRS analysis of blood could identify specific metabolic patterns in the rat depending on transplant kidney cold ischemia time that could be reversed by treatment with acetyl-cysteine [85,86]. Also, analysis of metabolite patterns in urine and serum using mass spectrometry technologies successfully distinguished between surgery and acute rejection in rats after kidney transplantation and could be used to monitor recovery [87]. Although metabonomics, as demonstrated in animal studies, shows promise for the development of diagnostic tools to detect and monitor immunologic events, transplant organ function and the tolerability of immunosuppressant drug regimens (toxicodynamic therapeutic drug monitoring), as of yet, only one study in transplant patients has been reported. Biochemical profiles in plasma and urine of 39 kidney graft patients were evaluated using ¹H-MRS [88]. The most relevant resonances correlated with renal function were citrate, trimethylamine-*N*-oxide (TMAO), alanine, and lactate. A resonance at 3.7 ppm was found related to cyclosporine toxicity when associated with elevated trimethylamine-*N*-oxide concentrations. However, the 3.7 ppm resonance was likely associated with polypropylene glycol, which is part of the cyclosporine Sandimmune formulation and therefore not an endogenous metabolite [88].

Similar to proteomics, a wide variety of analytical technologies has led to a variety of data types and standardization is required that will also allow for combination with genomic and proteomics data [89]. Standardization will have to include sample collection, preparation, and validation [89•]. In addition, as is already progressing in rats [90], a reference database providing a multivariate description of normal physiologic and biochemical variation of metabolites in blood, urine, and selected tissues in humans will be critical to distinguish metabolite patterns associated with pathologic processes, drug effect, or toxicity from normal variation.

8 Immunosuppression

Development of toxicodynamic monitoring strategies

Differentiation between chronic immunosuppressant toxicity and chronic rejection during the development of chronic renal dysfunction is important because it has a profound influence on therapeutic decisions. Chronic immunosuppressant toxicity is a far greater challenge than acute toxicity. As discussed above, pharmacokinetic therapeutic drug monitoring is not a viable strategy to avoid chronic immunosuppressant toxicity whereas toxicodynamic monitoring based on biomarkers appears as a more promising concept. Paradoxically, however, most clinical biomarker studies have focused mostly on acute rejection. Most clinical studies relevant for immunosuppressant toxicity have been carried out in the field of pharmacogenetics and toxicogenetics. However, the goal of most of those studies was to predict initial doses and thus to avoid early under-immunosuppression and acute toxicity. The importance for pharmacogenetic and toxicogenetic variants and long-term outcome has not yet been established. Although promising, proteomics technologies with one exception have not yet been applied to study immunosuppressant toxicity and metabonomics studies have mostly been limited to in-vitro and animal models.

One of the major shortcomings of most biomarker development efforts, and this is not limited to the field of transplantation, is that they are mostly descriptive and focus on the screening and identification of genomic, proteomic, and metabonomic patterns that are correlated with disease; however, very few programs have really followed through with translating those findings into patient care and developing the results into clinical diagnostic tools. The development of 'biomarkers' into diagnostic tools can be viewed and compared with drug development. Steps that are involved are screening, lead compound identification supported by data mining of the literature, development and validation of diagnostic tools (e.g. targeted gene and protein chips), clinical proof of concept trials, determination of sensitivity and specificity in multiple center trials, and depending on the country, approval by government agencies.

Another problem with today's common practice of biomarker discovery, especially in the fields of proteomics and metabolomics, is the lack of assay validation, such as establishing reproducibility, stability (long-term stability of stored samples, freeze-thaw stability, and in-process stability), and cross-validation with other laboratories. Validation is an absolute requirement for clinical chemistry, biochemistry, and drug analysis laboratories and is demanded by regulatory agencies such as the U.S. Federal Drug Agency [91]. The Human Proteome Organization has taken important first steps in this direction [73•]; similar efforts in the field of metabonomics are emerging [89•].

The major strength of systems biology in clinical research is that it allows for an 'unbiased' approach. This means data is recorded without a targeted search for known components and compounds which is always associated with selection and therefore loss of potentially valuable information. The combination of different 'omics' technologies has proven superior for the development of reliable biomarkers [3•]. Systems biology analysis always occurs in two stages: an instrumental and a bioinformatics phase. While the instrumental part is relatively well developed, searching and comparing data from different sources and filtering out the relevant differences is a challenge for which no satisfactory solution currently exists. Until this happens, a more realistic approach is the development of 'combinatorial biomarkers' – biomarker patterns that typically consist of five or more individual parameters – each of which usually must be changed twofold or more to yield a significant result [2•].

Conclusion

Biomarker identification and development using 'omics' and genetic strategies have the potential to yield new specific and sensitive diagnostic tools that will allow for:

- (1) Predicting tolerability of a immunosuppressive drug regimen and will allow for individualization of immunosuppressive therapy.
- (2) Monitoring allograft function and immunosuppressant toxicity.
- (3) Facilitating the development of a new generation of immunosuppressive drugs and immunosuppressive drug regimens by allowing more effective and faster pharmacodynamic and toxicodynamic screening and by providing new study endpoints for clinical development.

Overall, it is reasonable to expect that biomarker-based new diagnostic tools will significantly improve long-term outcome after transplantation.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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